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INHIBITION OF GENE EXPRESSION BY ALKYLPHOSPHONATE OLIGONUCLEOTIDE ANALOGS WITH RNase-LIKE ACTIVITY

FINAL REPORT

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19. ABSTRACT (Continue on reverse if necessary and identify by block number)
The technical objective was to synthesize an anti-viral oligonucleotide compound based on the sequence specificity of the target messenger RNA which would take advantage of the chemical instability of the m-RNA molecule. Anti-viral agents are required for control of AIDS in military populations.

Oligonucleotides have been developed which, with appropriate linkers, show promising results in vitro for lowering infectivity in cell cultures. Keywords:

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Final Report to US Army Medical Research and Development Command

1. Statement of problem under study

There is an urgent need to develop anti-viral compounds that can be used against the AIDS virus as well as other human viruses. It was decided to evaluate the so-called *antisense* approach, using oligonucleotides with the complementary base sequence to a selected viral gene. This involved the synthesis of several chemically modified analogs of oligonucleotides. There are two aspects of this approach, a) the need for a nuclease-resistant phosphodiester analog, and b) the possibility of attaching chemically reactive groups to the teminus of the oligo. In the latter case the group attached would be expected to destroy the cellular nucleic acid (mRNA) upon hybridization and therefore confer greater potency on any putative oligo-drug.

2. Background and Review

Shortly after this work was begun a report appeared that normal antisense oligos are active against HIV (1). However, in view of the anticipated degradation of these oligos in vivo by nucleases, a

nuclease-resistant analog was desirable. A methylphosphonate oligonucleotide analog (Fig. 1), had been proposed as an appropriate nuclease-resistant analog in elegant work by the group in Johns Hopkins University (2). I therefore anticipated using such an analog, but with the additional feature of attaching a chemical group to the oligo.

Intercalative groups such as fluorescent acridines had been attached to the 3' and 5' ends of oligos by Helene and co-workers (3). EDTA-attached oligos had also been synthesized by several groups, and these exhibited degradative activity on complementary nucleic acids, but only in the presence of high concentrations of Fe^{3+} (4). A Russian group has also synthesized oligos with alkylating groups attached (5).

3. Rationale Used in Current Study

It was decided to test the antisense approach against HIV, in collaboration with Dr. Samuel Broder and his group in NCI. For this purpose a specific conserved sequence in the HIV genome was identified, and normal, methylphosphonate (PM) and phosphorothioate (PS) oligodeoxynucleotides were synthesized in the automatic synthesizer. The improvements in automated synthesis were accomplished in collaboration with Drs. Kazuo Shinozuka and Gerald Zon. It was found that the PS oligos were reproducibly effective against HIV, while the normal and PM oligos were not (*1,2*). These results were obtained at the beginning of the contract, and necessitated a change of focus from the PM to the PS oligos. The

contract enabled the setting up of an automatic DNA synthesizer in the lab of the contractee, and the employment of a technician and a technician to run it.

Since covalently attached groups had never been reported for an oligo analog of this kind, it was decided to start with known groups, such as acridine, in order to compare the results with those of Helene and co-workers on normal oligos (3). These fluorescent oligos could then be used to follow cellular uptake as fluorescently tagged oligos. It was also decided to attempt to synthesize oligos with an attached reactive drug type group, and anthraquinone was chosen for this purpose, since it is a radical-producing group similar to the well-known anti-cancer drugs, adriamycin and bleomycin. Finally, it was decided to attach imidazole to the oligo in order to attempt to confer specific RNase activity, similar to that found in RNase enzymes (6). A chemist was employed under the contract to sythesize these analogs.

4. Experimental Methods

HIV assays were carried out by Dr. M. Matsukura as described in the literature (7). Automated syntheses were carried out by the standard stepwise addition of a nucleotide as a phosphoramidite, except that for the PS analogs, sulfurization rather than oxidation was employed (*3*). Phosphous-31 NMR was routinely used to monitor the preparation of the PS analogs.

Chemical syntheses were carried out as far as possible in the automatic synthesizer. This was considered the most efficient means

to obtain large quantities of product for biological studies. This necessitated the preparation of novel 5'-linked phosphoramidites (details of syntheses will be found in the relevant publications, ***4-6***). This was necessary because the oligo is synthesized from the 5' end with the 3' end attached to the solid support. Attachment of the group after synthesis and purification of the oligo is not as efficient. Proton NMR was used to characterize the linked compounds.

5. Results

a. Anti-HIV activity - sequence non-specific inhibition.

The suprising finding of the initial experiments (***1***) was that one of the controls, a PS-homo-oligomer of deoxycytidine (S-dC28) was the most effective anti-HIV agent, i.e. 100% protection of ATH8 cells against the cytopathic effect of HIV-1 at < 1 μM concentration, without any toxicity (Fig. 1). It was clearly shown that there is a base composition (G,C>>A,T) and a length effect (28>21>14>>5). There was no evidence of significant degradation of these compounds during the assay period of up to a week.

It was also shown that there is inhibition of de novo viral DNA synthesis by these PS-oligos (***1, 2***). This lead us to test for inhibition of reverse transcriptase, and it was found that S-dC28 has a Ki 200-fold smaller than the normal oligo (***7***). Although this may be related to the sequence non-specific activity of these compounds, it was also found that these compounds inhibit other lentiviruses, but not other retroviruses such as MuLV (***8***). Similarly, a pattern of selective

inhibition of some human polymerases and herpes simplex virus polymerases have been observed (*9*). At present we cannot be sure of the precise mechanism(s) for the sequence non-specific inhibition of acute HIV infections by S-oligos.

b. Anti-HIV activity - sequence-specific inhibition

Since the object of this work was to demonstrate an antisense effect of a nuclease-resistant oligo we tested the anti-rev (a-rev) sequence against a chronically infected T-cell (H9), and looked for viral gene products (gag protein). A dose dependent inhibition was observed, and this specific for the antisense sequence, neither the sense, nor a random sequence, nor a methylated antisense sequence, nor a normal antisense oligo worked (*10*). It is our belief that this is the only compound yet found that inhibits HIV in chronically infected T-cells. Since the rev gene is a regulatory gene, it was expected and found that there is an effect on the mRNA profile of HIV by the antisense oligo. Whether the mechanism of this antisense effect is due to an RNase H activity (*11*) is not known.

c. Cellular uptake.

Since it has been considered unlikely that multiply charged biopolymers like oligos could cross cell membranes, it was decided to use the fluorescently tagged oligos to follow cellular uptake. This was done incollaboration with Dr. Len Neckers and his group in NCI, using the fluorescent activated cell sorting technique (FACS). It was

found that oligos do indeed enter HL60 cells rather rapidly, the results depending on the length (Fig. 3). The S-oligos were found to be much more slowly taken up than the normals (*3,12*). This probably acounts for the poor inhibition of myc protein expression seen over periods of hours for the S-oligos, that was comparable to the normal when they were delivered in liposomes (*12*).

In subsequent work the surface proteins of HL60 were radioactively labelled, and following cell lysis and solubilization, were passed through a column containing an oligo attached to the beads. Only a single band of 80 kD stuck to the column, and this has been tentatively identified as a cellular receptor protein for oligos, facilitating their intracellular transport (*13*).

d. Anti-HIV activity of linked oligos

The 5'-linked acridine and anthraquinone a-rev S-oligos were tested in the ATH8 assay in comparison with the non-linked oligo. Both were found to be more effective. Namely the attachment of these groups produces a more potent drug, in the case of the anthraquinone-linked compound a 10-fold improvement in dose effect was observed (*14*).

6. Discussion and Conclusions:

The results of this project have been essentially successful:
a) A category of nuclease-resistant oligo analogs (the PS analog) have been identified as anti-HIV agents.

b) The antisense effect of a sequence specific anti-rev sequence has been shown to occur in vitro in chronically infected H9 cells.

c) The cellular uptake of this category of compound has been investigated, and shown to occur.

d) S-oligos with covalently attached reactive groups have been found to be more potent in their anti-HIV activity than the non-linked oligo.

Two areas await further research. The imidazoyl linked compounds could not be synthesized, even though three different approaches were tried over a period of two years. Thus, the development of a more specific hydrolytic attached group remains to be achieved.

The potential in vivo effects of these oligo analogs has not been tested. However, large (gram) quantities are being supplied to NCI in order that the required in vivo toxicology tests can be carried out on mice, before potential use in humans is possible. These studies are beyond the scope of the contract.

The results of this work, involving synthesis of novel compounds, and inter-disciplinary studies of several kinds, have been extensive. It may be said that it has contributed to the development of a new form of chemotherapy or of pharmacology (8-10).

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*(note: citations in **bold italic** refer to attached bibliography)*

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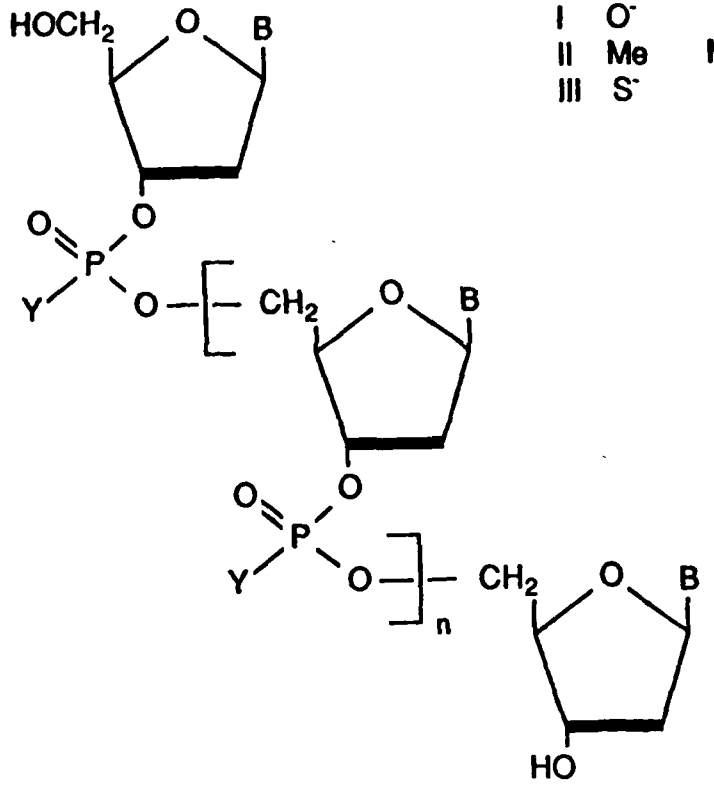
Personnel supported by the contract:

Chrisanthe Subasinghe, B.A. - August, 1987-June, 1989.

Kenya Mori, Ph.D. - December, 1987-March, 1989.

Structure of Oligodeoxynucleotides



	Y	
I	O^-	PHOSPHATE
II	Me	METHYLPHOSPHONATE
III	S^-	PHOSPHOROTHIOATE

FIGURE 1

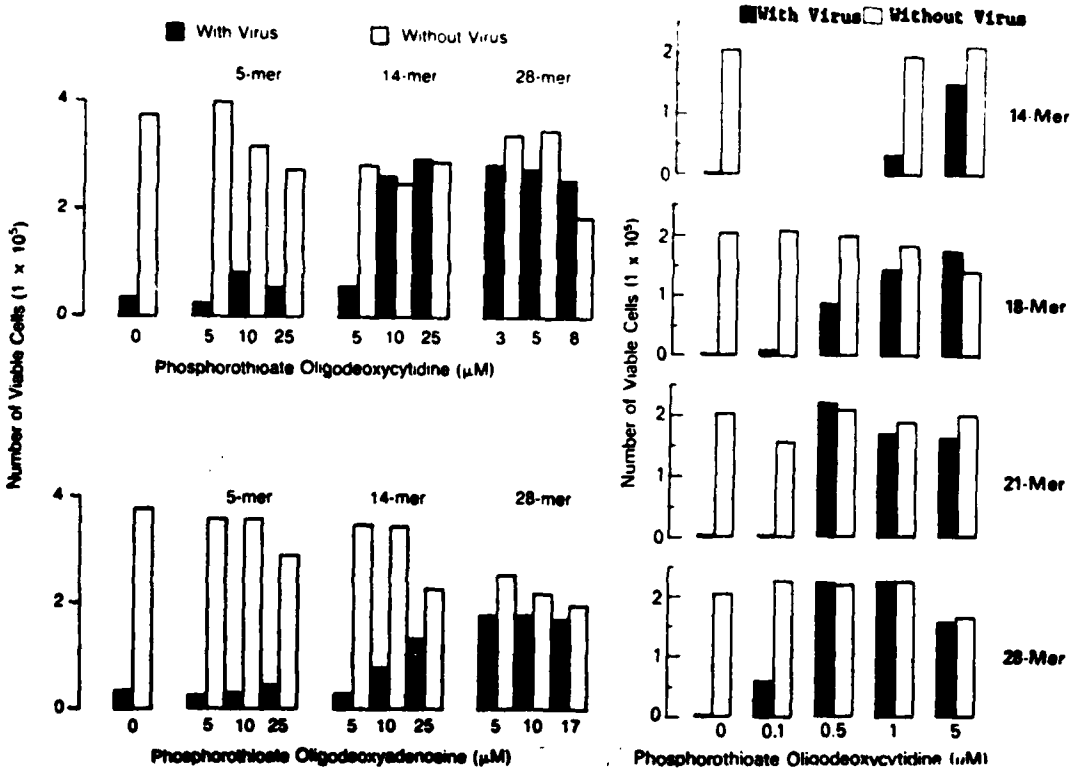


FIGURE 2

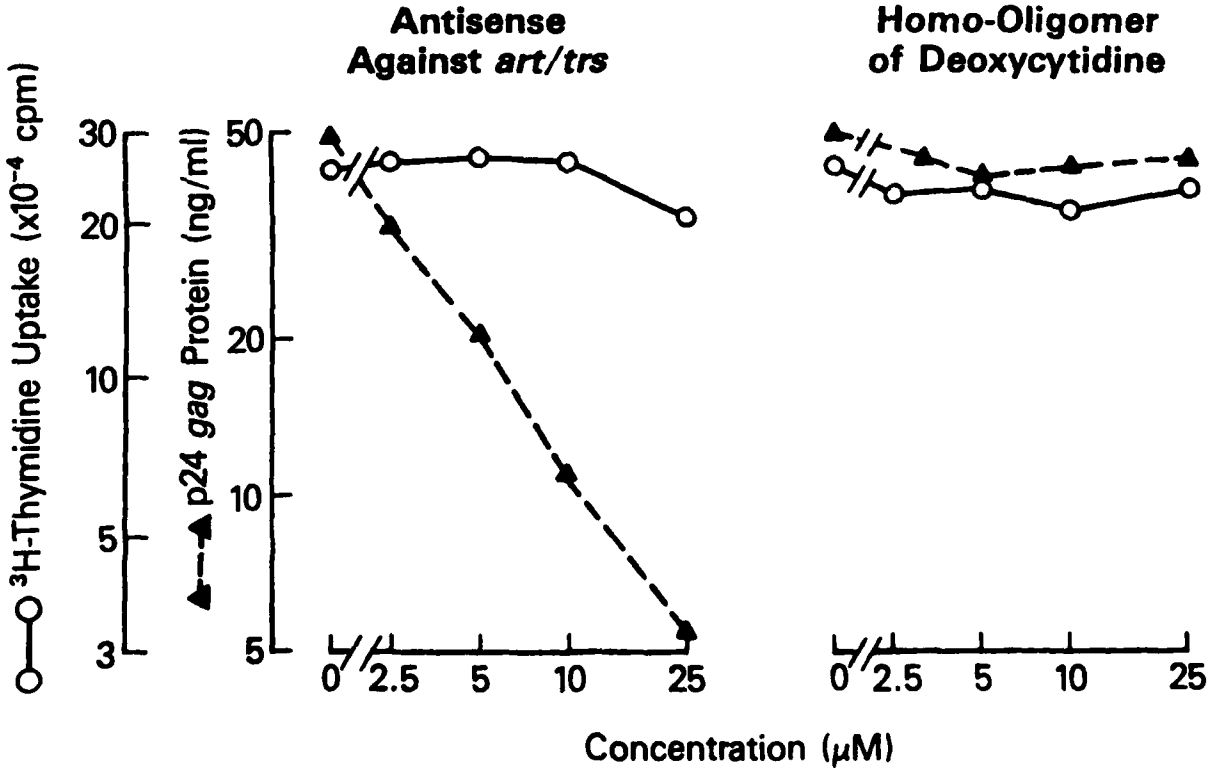


FIGURE 3

A.

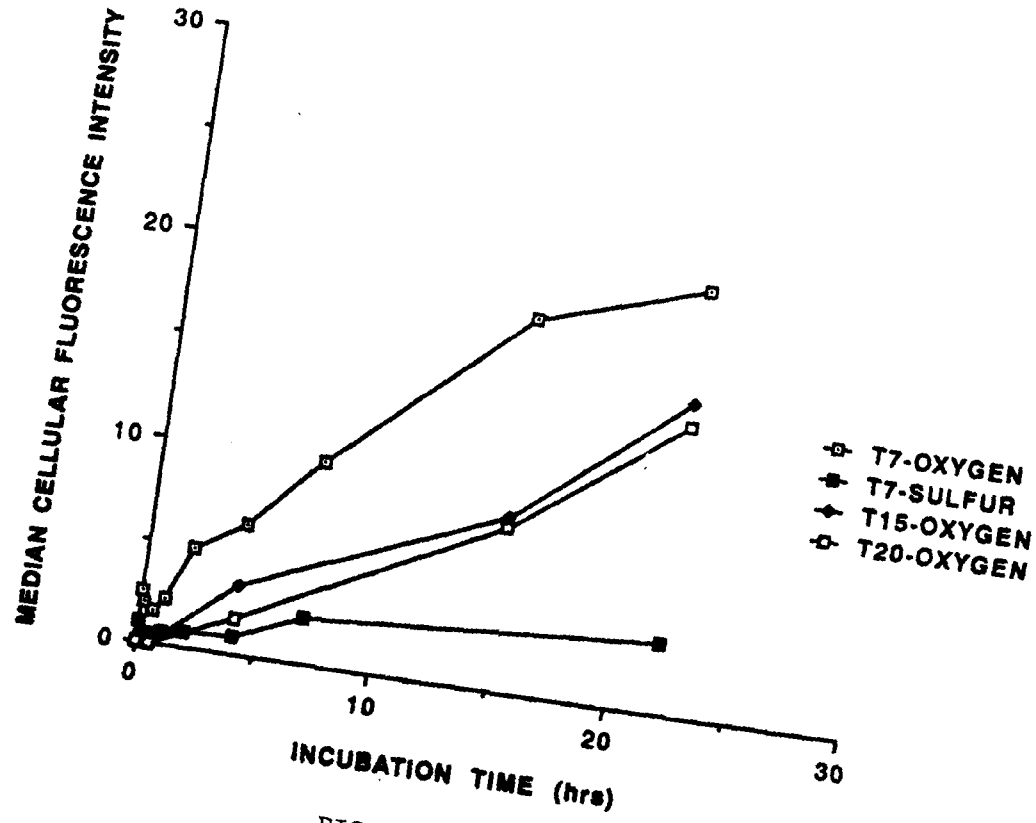

MEDIAN CELLULAR FLUORESCENCE INTENSITY
30
20
10
0
0
10
20
30
INCUBATION TIME (hrs)
T7-OXYGEN
T7-SULFUR
T15-OXYGEN
T20-OXYGEN

FIGURE 4